JOURNAL OF VIROLOGY, Sept. 1994, p. 5638–5647
0022-538X/94/$04.00+0

Vol. 68, No. 9

Basic Helix-Loop-Helix Proteins in Murine Type C Retrovirus Transcriptional Regulation

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Received 24 March 1994/Accepted 7 June 1994

E boxes, recognition sequences for basic helix-loop-helix (bHLH) transcription factors, are detected in the enhancer and promoter regions of several murine type C retroviruses. Here we show that ALF1, a member of bHLH protein family of transcription factors, in vitro binds with differing affinities to distinct E-box sequences found in the U3 regulatory regions of Friend, Moloney, SL3-3, and Akv murine leukemia viruses (MLVs) as well as Friend spleen focus-forming virus (SFFV). In NIH 3T3 fibroblasts, ALF1 overexpression elevated transcription from the U3 region of Moloney MLV and the complete long terminal repeat regions of Friend SFFV, Akv MLV, and SL3-3 MLV but neither from the U3 region nor from the complete long terminal repeat of Friend MLV. Introduction of mutations in the Akv MLV E boxes showed the E-box *cis* elements to be required for the function of ALF1 as a transcription factor. ALF1 and the glucocorticoid receptor, with overlapping DNA binding sequences, did not act synergistically with respect to transcriptional *trans* activation of expression from the Akv MLV promoter-enhancer region. We conclude that ALF1 in vivo may be an important transcription regulator for Akv, SL3-3, and Moloney MLVs as well as for Friend SFFV.

Transcription of murine type C retroviruses is regulated by an array of transcription factors, which bind to their corresponding regulatory *cis* elements in the U3 enhancer (38, 39, 60, 62). The murine type C retrovirus family includes murine leukemia viruses (MLVs), sarcoma viruses, spleen focus-forming viruses (SFFVs), and mink cell focus-forming viruses (MCFVs). Sarcoma viruses, SFFVs, and MCFVs, formed by recombination events involving MLV sequences, are pathogenic agents (69). Transcriptional regulatory sequences in the long terminal repeat (LTR) U3 region of MLVs are determinants for pathogenesis (10, 11, 15, 16, 20, 33, 35, 54, 61). For example, the capacity of the erythroid cell leukemogenic Friend MLV and the T-cell leukemogenic Moloney MLV to induce the respective diseases is determined by the U3 enhancer sequences (10, 11, 25, 35). Likewise, major determinants for the difference between the chronic T-cell leukemogenic SL3-3 MLV and the weakly leukemogenic Akv MLV are localized in the U3 enhancer sequences (7, 33).

The E-box consensus sequence (NCANNTGN) overlaps a glucocorticoid response element (GRE) in the U3 region of murine type C retroviruses (8, 14, 18, 32, 47). GREs may facilitate transcriptional activation by steroid hormones (8, 9, 40). The GRE-overlapping E box has the conserved sequence ACAGATGG (reference 18 and references therein; 14, 47). Of 30 murine type C retrovirus sequences aligned by Golemis et al. (18), only radiation leukemia virus and Friend MLV lack this particular E-box motif in their U3 regions (reference 18 and references therein; 27, 30). Introduction of mutations in the three Moloney MLV U3 GREs, destroying the overlapping E boxes, increased the latency period of disease induction twofold (61). In an SL3-3 MLV U3 construct, mutation of the equivalent E boxes decreased transcription twofold in lymphoid cell lines (14). Besides the E box overlapping the GRE, other E-box elements can be identified in MLV U3 regions. For example, an E box is located at the LVa site in Moloney MLV (62).

The human basic helix-loop-helix (bHLH) DNA-binding protein SEF2-1 has been cDNA cloned for binding to the E box overlapping the GRE in the SL3-3 MLV enhancer, and the murine bHLH proteins ALF1 and ALF2 have been cDNA cloned for binding to the equivalent E boxes in the Akv MLV enhancer (14, 47). The analog to SEF2-1 is E2-2 (22). The human analog to ALF1 is called HEB or HTF4, and the rat analog is REB (24, 29, 74). ALF2 is identical to A1, and the human and rat analogs are E47 and Pan-1, respectively (42, 46, 67). Presumably as a result of alternative splicing, *ALF1* mRNA exists in two forms resulting in two protein products differing by a 24-amino-acid insertion in ALF1B (76 kDa) relative to ALF1A (73 kDa) (47). Both ALF1A and ALF1B have the ability to function as transcriptional activators (47). The human ALF1A analog HEB has affinity to E boxes in the human immunodeficiency virus type 1 promoter, CD4 enhancer, and immunoglobulin enhancers (24, 57, 75).

ALF1, ALF2, and the human bHLH proteins HEB, E12/E47/E2-5, and E2-2/SEF2-1 form a very homologous group called the class A bHLH transcription factors (14, 22, 24, 42, 43, 47, 74). Class A bHLH proteins are involved in the regulation of tissue-specific gene expression as either homodimers, intraclass heterodimers, or heterodimers with bHLH proteins from class B, such as MyoD1 and myogenin (2, 24, 31, 43, 64, 68). Moreover, class A bHLH proteins are capable of forming heterodimers with the Id proteins, which are helix-loop-helix proteins lacking the basic DNA binding region (4, 12, 65). Id proteins are dominant negative for transcriptional *trans* activation, and the expression of Id proteins is inversely correlated with bHLH protein activity (4, 28, 52, 65, 70).

In this study, we show that ALF1 has affinity for various E-box consensus motifs found in U3 regulatory regions of Friend, Moloney, SL3-3, and Akv MLVs as well as the polycythemia-inducing strain of Friend SFFV (SFFVp). We demonstrate that ALF1 *trans* activates transcription of Moloney, Akv, and SL3-3 MLVs as well as Friend SFFVp, whereas Friend MLV is not activated. Introduction of mutations in all

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Akv MLV U3 E boxes verifies that intact E-box elements are essential for transcriptional *trans* activation by ALF1. Our analyses suggest that ALF1 in vivo may be an important transcriptional *trans* activator of murine type C retroviruses with E-box sequences in their enhancer regions, such as Akv, SL3-3, and Moloney MLVs as well as Friend SFFVp.

MATERIALS AND METHODS

Bacterial strains and plasmids. Standard molecular biology methods were used (3, 56). For maintenance of plasmids, we used *Escherichia coli* K803 and DH-1 (21, 72).

Cloning of cDNA encoding full-length ALF1A and ALF1B was previously described (47). Eukaryotic expression plasmids having no cDNA or the ALF1 cDNA variants under regulation of the EF-1α promoter were generated as described previously (50).

pMoCAT, kindly provided by N. A. Speck (Department of Biochemistry, Dartmouth Medical School, Hanover, N.H.), was generated by insertion of Moloney MLV U3 and R sequences from the *Sau*3AI site to the *Kpn*I site into pUCCAT as described previously (62). pMo(GREbc)CAT and pMo(GRE abc)CAT with mutated E boxes were generated from pMoCAT by site-directed mutageneses as described previously (62). pMoP-CAT was generated by insertion of Moloney MLV U3 and R sequences from the *Xba*I site to the *Kpn*I site in pUCCAT as described previously (62). The Moloney MLV *cat* gene constructs were kindly donated by N. A. Speck. pLFrCAT was generated from the Friend-57 clone by insertion of U3 and R sequences (*Pst*I to *Kpn*I) into pSV2CAT (59). pLMoCAT was generated equivalently, using instead a Moloney MLV U3/R fragment (*Kpn*I to *Afl*II). The *Afl*II site in Moloney MLV is located precisely in the same position as the *Pst*I site in Friend MLV. pLFrCAT and pLMoCAT (L in plasmid names added for reasons related to nomenclature) were kindly donated by J. Lenz (Albert Einstein College of Medicine, Bronx, N.Y.). pFrL-TRCAT and pFrSFFVpCAT include Friend and Friend SFFVa sequences, respectively, from *env* to *pol* with a complete LTR as described previously (63). These two constructs were kindly donated by D. Kabat (Department of Biochemistry, School of Medicine, The Oregon Health Sciences University, Portland). pAKV6CAT was generated by insertion of a complete Akv MLV LTR with flanking sequences into pSV2CAT (51). pSL3-3CAT was generated by exchanging a U3/R fragment (*Pst*I to *Kpn*I) from pAKV6CAT with the equivalent SL3-3 MLV fragment (51). The construct pD19CAT with an Akv MLV promoter deletion was generated as described previously (49). p1-99CAT with one Akv MLV enhancer repeat was generated from pAKV6CAT by deletion of a 99-bp *Apa*I fragment (37).

Construction of the pAKV(Eabc)CAT construct. For the generation of E-box mutations in the Akv MLV LTR, we used an oligonucleotide with a mutation in the promoter E box (5231; CCCCGGT*CATCTT*GGGAACCTTG; the E box is in italics, and the mutation is underlined) and a 139-nucleotide oligonucleotide with mutations in both enhancer E boxes (5229; CGTCTAGAGCGGGCCCCGGCCCAGGGCCAAG AA*AAGATG*GTCCCCAGAAACAGAGAGGCTGGAAA GTACCGGGACTAGGGCCAAACAGGATATCTGTGGT CAAGCACTAGGGCCCCGGCCCAGGGCCAAGAA*AAG ATG*G; non-Akv MLV sequences are underlined, and mutated E boxes are in italics) in PCR (94°C for 1 min, 50°C for 1 min, and 73°C for 1 min for 20 cycles, using *Taq* polymerase [Stratagene]) in a Hybaid OmniGene temperature cycler. The p1-99CAT construct was used as the template. Oligonucleotide 5229 covers four bases 3′ from the repeat Akv MLV E box and includes in the 5′ end a 14-bp PCR tag nonhomologous to Akv MLV sequences; an additional 20 PCR cycles were carried out with oligonucleotide primer 5230 (5230; CGTCTA-GAGCGGGC) with sequence equivalent to that of the PCR tag. The generated PCR product was used as primer in PCR with an oligonucleotide primer corresponding to Akv MLV sequences at the R region (1166; GGCTTTATTGGATA-CACGGGTACCCGGGCG) (94°C for 1 min, 55°C for 1 min, and 73°C for 1 min for 20 cycles). The PCR product was digested with *Kpn*I and partially digested with *Apa*I, and the longest *Apa*I-*Kpn*I fragment (343 bp) was cloned into the corresponding sites of p1-99CAT, thereby generating pA-KV(Eabc)CAT, in which all of the Akv MLV E boxes are mutated on an intact Akv MLV LTR background. The presence of specific mutations was verified by using an Applied Biosystems *Taq* sequencing kit on an Applied Biosystems 373A sequencer.

TABLE 1. Sequences of oligonucleotides used in EMSA and filter binding assays

Oligonucleotide	Sequence[a]
E_{GRE}	AAATTTCAAG**AACAGATGGT**CCCCAGAAATAGCT
E_{mut}	AAATTTCAAG**AAAAGATGGT**CCCCAGAAATAGCT
$E_{A/S}$	AAATTTCAAG**CCCAGATGAC**CCCCAGAAATAGCT
$E_{M5'}$	AAATTTCAAG**CCCACCTGTA**CCCCAGAAATAGCT
E_{LVa}	AAATTTCAAG**AACAGCTGAA**CCCCAGAAATAGCT
E_{MPr}	AAATTTCAAG**CCCAGATGCG**CCCCAGAAATAGCT
$E_{M/F}$	AAATTTCAAG**ATCAGATGTT**CCCCAGAAATAGCT
R_{Filt}[b]	AGCTATTTCTGGGG
R_{EMSA}[c]	GTTTAGCTATTTCTGGGGACCATCT

[a] Cassette regions including the E-box motifs are given in boldface, and the E-box core residues are underlined.
[b] Used for generation of probes for filter binding assays.
[c] Used for generation of probes for EMSA.

Nuclear extracts, *E. coli*-expressed ALF1 proteins, and electrophoretic mobility shift assay (EMSA). NIH 3T3 fibroblast nuclear extracts were prepared as described previously (1). *E. coli*-expressed complete ALF1A and ALF1B proteins were produced and purified as described previously (47).

EMSA probes were prepared by annealing either oligonucleotides E_{GRE} and R_{EMSA} or oligonucleotides E_{mut} and R_{EMSA} (Table 1) and filling in staggered ends with Klenow polymerase (Amersham) in the presence of [α-^{32}P]dATP (3,000 Ci/mmol; Amersham). Probes were purified by 10% native polyacrylamide gel electrophoresis (PAGE). The competing oligonucleotides were made by similar procedures.

EMSA using *E. coli*-expressed ALF1 was done essentially as described previously (47) except that Tween 20 was included in EMSA reaction mixture to a final concentration of 0.25%. ^{32}P-labeled probe (2×10^4 cpm) and unlabeled competitors were mixed before addition of ALF1 (2 μl, approximately 10 ng) and then incubated for 15 min at room temperature. Samples were loaded on 0.5× Tris-borate-EDTA (pH 7.5)–4% acrylamide EMSA gels, run for 2.5 h at 200 V, dried, and exposed with intensifying screens. In EMSA using nuclear extracts, 2×10^4 cpm of ^{32}P-labeled probe, 1.0 μg of poly(dI-dC) · poly(dI-dC), and competitors were mixed in the same buffer as described previously (47) except that detergents were omitted. Then 3 μg of nuclear extract was added, and the mixture was incubated for 15 min at room temperature before being subjected to electrophoresis.

Filter binding assay. Thirty picomoles of oligonucleotide R_{Filt} (Table 1) was 5′ labeled with [γ-^{32}P]dATP (7,000 Ci/mmol; ICN), using T4 polynucleotide kinase. Three-picomole fractions of labeled R_{Filt} were annealed with a 100-fold excess of various E-box-containing 34-nucleotide oligonucleotides.

All E-box oligonucleotides have a common 3′ end complementary to oligonucleotide R_{Filt} and a common 5′ end flanking a variable 10-nucleotide cassette region (Table 1). By low-temperature PCR (80°C for 1 min, 42°C for 1 min, and 62°C for 1.5 min for 15 cycles, using *Taq* polymerase [Stratagene]), the oligonucleotides were made double stranded. The double-stranded ^{32}P-labeled oligonucleotides were purified by native 10% PAGE. The integrity of generated E-box probes was checked by denaturing 12% PAGE. Two microliters (approximately 10 ng) of *E. coli*-produced and purified ALF1B was dotted on 1-cm^2 cellulose nitrate membranes (BA85; Schleicher & Schuell) and briefly dried at room temperature. Filters were washed for 30 min at 4°C in 1 ml of 1× SW (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.2], 100 mM KCl, 10% glycerol, 1 mM dithiothreitol, 0.25% Tween 20)–3% nonfat dry milk and then washed in 1× SWM (SW, 0.25% nonfat dry milk). From 1 × 10^3 to 1,024 × 10^3 cpm of probe was added in 500 μl of 1× SWM to the filters, which were incubated for 3 h at 4°C. Filters were washed three times for 15 min each time in 1× SWM, briefly dried, and exposed to film. The amount of probe retained on filters was estimated by liquid scintillation counting. Each experiment included all probes to ensure comparability.

DNA transfections. NIH 3T3 mouse fibroblast cells were maintained in Dulbecco's modified Eagle's medium–10% calf newborn serum. Cells were seeded to 5 × 10^5 cells per 100-mm-diameter plate 24 h before transfection. Medium was replaced 4 h prior to transfection by calcium phosphate-mediated precipitation as described previously (37). Briefly, each precipitate included 0.5 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid (2 μg for the various pMo-CAT constructs), 2.0 μg of either ALF1A (or ALF1B) expression vector or 2.0 μg of the empty expression vector, and 8 μg of pCH110, an internal standard with β-galactosidase expressed from the simian virus 40 promoter (19). β-Galactosidase activity was assayed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). CAT assays were done as described previously, and conversion was normalized to β-galactosidase activity to correct for variation in transfection efficiency (37). Dexamethasone induction (final concentration, 1 μM) was done 24 h after transfection as described previously (8).

RESULTS

ALF1 interacts with GRE-overlapping E boxes. U3 regions of several murine type C retroviruses contain E-box consensus sequences (NCANNTGN) overlapping putative GREs (14, 18, 32, 47). This type of E box has the conserved sequence ACAGATGG, hereafter abbreviated E_{GRE}. Of 30 murine type C retrovirus sequences aligned by Golemis et al. (18), only radiation leukemia virus and Friend MLV lack the E_{GRE} motif in the U3 regions (reference 18 and references therein; 27, 30). The murine type C retroviruses with the E_{GRE} used in our analyses include Akv MLV, with one E_{GRE} in each of the direct repeats, and SL3-3 and Moloney MLVs, with three E_{GRE}s (Fig. 1) (34, 58, 66). As noted, Friend MLV is depleted of E_{GRE} motifs in the U3 region, while the Friend SFFVp derived from Friend MLV contains one E_{GRE} (Fig. 1) (13, 30).

By EMSA, we analyzed binding of the ALF1 bHLH proteins to the E_{GRE} sequence. As the source of ALF1 for EMSA, we used ALF1A or ALF1B produced in *E. coli* and purified as described previously (47). EMSA probes were double-stranded oligonucleotides with either the authentic E_{GRE} (ACA GATGG) or a mutated E box, E_{mut} (AAAGATGG; the modified base is underlined) (Table 1). As competitors, we used equivalent unlabeled oligonucleotides. As shown in Fig. 2, both ALF1A and ALF1B formed sequence-specific complexes with the E_{GRE} oligonucleotide probe (Fig. 2A and B for ALF1A and ALF1B, respectively). Consequently, ALF1 proteins bind the E_{GRE} sequence found in multiple murine type C retroviruses with requirement of the intact E-box motif. We did not detect any significant difference between ALF1A and ALF1B in our E_{GRE} EMSA analysis.

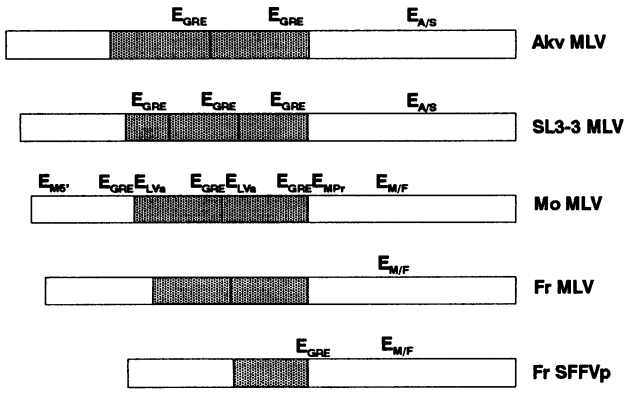

FIG. 1. Structures of U3 regions and positions of E boxes in Akv, SL3-3, Moloney, and Friend MLVs and Friend SFFVp. Sequences of E boxes are as follows: E_{GRE}, ACAGATGG; $E_{A/S}$, CCAGATGA; $E_{M5'}$, CCACCTGT; E_{LVa}, ACAGCTGA; E_{MPr}, CCAGATGC; and $E_{M/F}$, TCAGATGT. Shaded areas indicate enhancer repeat structures. The U3 regions from Akv MLV and SL3-3 MLV are identical except for a nucleotide residue insertion at position −418 and the sequences in the repeats (34, 66). Akv MLV has a 99-bp tandem repeat, and SL3-3 MLV has a 72-bp tandem repeat preceded by a 34-bp partial repeat segment. Moloney MLV has a 75-bp tandem repeat (58). Friend MLV has a 65-bp tandem repeat (30). The Friend SFFVp U3 region, relative to the parental Friend MLV U3 region, contains point mutations and lacks one repeat (13).

ALF1 E_{GRE} binding activity is not detectable in NIH 3T3 cells. Protein complexes that bind to E-box probes and in

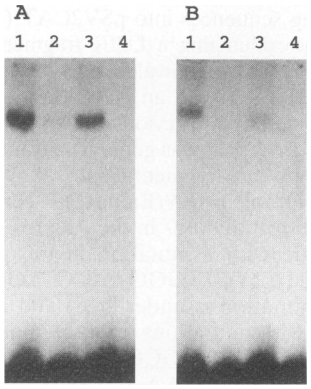

FIG. 2. ALF1 binds the murine type C retrovirus GRE-overlapping E box E_{GRE}. ALF1A (A) or ALF1B (B) was expressed in *E. coli* and purified from inclusion bodies. EMSA was done with oligonucleotide probes corresponding Akv MLV enhancer sequences including the E_{GRE} (lanes 1 to 3) or a mutated E_{GRE} (lanes 4). Samples include ALF1 and ^{32}P-labeled wild-type oligonucleotide probe (lanes 1), 10-fold excess of wild-type E-box oligonucleotide competitors (lanes 2), 10-fold excess of E-box mutated oligonucleotide competitors (lanes 3), or ALF1 and a ^{32}P-labeled E-box mutated oligonucleotide probe (lanes 4).

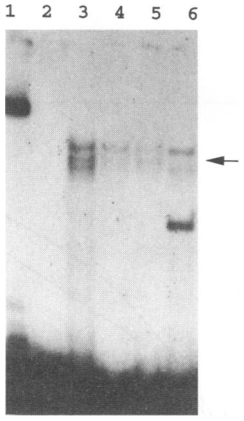

FIG. 3. Cellular ALF1 DNA-binding activity is not detectable in NIH 3T3 cells. EMSA was done with double-stranded oligonucleotides including the E_{GRE} (lanes 1 to 5) or a mutated E_{GRE} (lane 6). Samples include *E. coli*-produced and purified ALF1A (lane 1), ^{32}P-labeled E_{GRE} oligonucleotide probe in the absence of ALF1 (lane 2), NIH 3T3 nuclear extract (lane 3), NIH 3T3 extract and 10-fold excess of wild-type oligonucleotide competitors (lane 4), NIH 3T3 extract and 10-fold excess of E-box mutated oligonucleotide competitors (lane 5), and NIH 3T3 nuclear extract and E_{mut} oligonucleotide probe (lane 6). An NIH 3T3 cell E-box region-binding protein complex not identical to the homodimeric ALF1 complex is indicated by an arrow.

EMSA behave like the *E. coli*-generated homodimeric class A bHLH proteins have been identified in nuclear extracts from several cell lines (26, 57). For example, protein complexes specifically interacting with the E_{GRE} have been identified in lymphoid cell lines (14).

By EMSA, we examined whether proteins with the same EMSA characteristics as homodimeric ALF1 exist in NIH 3T3 cells. NIH 3T3 nuclear extract exhibited formation of at least three different protein complexes, using oligonucleotide probes with an E_{GRE} motif (Fig. 3). The formation of one of the protein complexes, indicated with an arrow in Fig. 3, was dependent on an intact E-box sequence (Fig. 3, lanes 3 to 6). Consequently, this band may represent a specific E-box-binding protein complex. However, in the NIH 3T3 extract, it was not possible to detect a DNA-protein complex migrating like the DNA-ALF1 complex (Fig. 3; compare lanes 1 and 3). Thus, in NIH 3T3 cells, an E_{GRE}-binding protein complex composed of homodimeric ALF1 is not detectable. This result does not exclude the possibility that ALF1 is expressed as a protein in NIH 3T3 cells; ALF1 may exist in a non-DNA-binding protein complex with Id protein (4, 12, 65). We note that the mRNA for ALF1 is expressed in NIH 3T3 fibroblasts (47).

ALF1 binds different E boxes with variable affinities. Apart from E boxes in the putative GREs of Friend SFFVp and in Akv, SL3-3, and Moloney MLVs, other U3 E-box motifs can be identified in these viruses (Fig. 1). All identified E boxes have a G residue at position −1 relative to the symmetric axis in the E-box core (NCAGNTGN). A G residue in this position has previously been shown to generate an E box, to which class A bHLH proteins bind (6, 48, 64) (data not shown). In methylation interference assays using ALF1 or other class A bHLH proteins, the G residue in the −1 position is found to be sensitive to methylation (43) (data not shown). Class C bHLH proteins such as Myc prefer a C residue in the −1 position (5). $E_{M5'}$ is a putative binding site for both class A and class C bHLH proteins (Table 1).

We wanted to examine the relative affinities for binding of ALF1 to the different U3 E boxes. Double-stranded probes with authentic or mutated U3 E boxes in an Akv MLV sequence background were generated by using a common complementary ^{32}P-labeled primer (Table 1). Double-stranded probes were of the same length and labeled to the same specific activity (Fig. 4A). Purified ALF1 was immobilized on nitrocellulose filters, and probes were added in amounts varying from 1×10^3 to $1{,}024 \times 10^3$ cpm. The amounts of retained probe were visualized by exposing to film (Fig. 4B) and measured by liquid scintillation counting. The relationship between DNA in the reaction and bound DNA is shown graphically in Fig. 4C. Thereby, ALF1's abilities to bind the different probes can be compared and used as an indication of relative binding affinity. The E-box preferences for ALF1 are, in relative order, $(E_{GRE} = E_{M5'}) > (E_{MPr} = E_{LVa} = E_{M/F}) > E_{A/S} > E_{mut}$. Thus, for the Akv and SL3-3 MLV U3 regions, the E_{GRE}s in the sequence repeat have the highest affinity for ALF1. Besides, ALF1 has a low-affinity binding site, $E_{A/S}$, located in the upstream promoter. In the Moloney MLV U3 region $E_{M5'}$, E_{MPr}, E_{LVa}, and $E_{M/F}$ constitute ALF1 binding sites in addition to the E_{GRE}s. Friend MLV U3 has the $E_{M/F}$ to which ALF1 binds, while the Friend MLV-derived Friend SFFVp has acquired, besides $E_{M/F}$, the high-affinity E_{GRE} ALF1 binding site.

ALF1 *trans* activates SL3-3 and Akv MLV transcription and requires the existence of E boxes. We have not observed any significant differences in the transcriptional *trans*-regulatory functions of ALF1A and ALF1B (47) (data not shown). In the following experiments, ALF1A was used in expression studies unless indicated otherwise. For in vivo examination of the *trans*-regulatory function of ALF1, we cloned the coding region into an EF-1α promoter-regulated eukaryotic expression vector for cotransfection analyses (41, 50). We used plasmids pSL3-3CAT and pAKV6CAT, carrying complete LTRs of SL3-3 MLV and Akv MLV, respectively, as reporters (51). In NIH 3T3 cells, the CAT expression from pSL3-3CAT constituted approximately 20% of the CAT expression from pAKV6CAT (Table 2). As previously observed, this result shows that the Akv MLV promoter-enhancer is stronger than the SL3-3 MLV promoter-enhancer in NIH 3T3 cells (7). Overexpression of ALF1 resulted in threefold elevation of CAT expression from pAKV6CAT, while CAT expression from pSL3-3CAT was elevated roughly 10-fold by ALF1 expression (Table 2). Therefore, ALF1 has the capacity to *trans* activate transcription on the basis of the relatively intact MLV promoter-enhancer sequences in a complete LTR. In the presence of ALF1, the absolute transcription rates were the same from Akv and SL3-3 MLV promoter and enhancer regions. The level of transcription is independent of the strength of the promoter and enhancer regions without ALF1 overexpression.

To examine whether the ALF1 *trans*-activator function could be linked to E-box sequences in the enhancer and promoter regions of our reporter constructs, we analyzed versions of our pAKV6CAT construct with mutations abolishing E boxes (Fig. 5). Plasmid pD19CAT has a deletion in the U3 region from positions −119 to −314, including all three Akv MLV U3 E boxes (numbers are relative to the transcription initiation site) (Fig. 5) (49). As a consequence of the deletion, a new consensus E box (GCACATGA) was generated, but because of ALF1 E-box preferences, the E box should not constitute an efficient ALF1 binding site (data not shown). The CAT expression from pD19CAT was reduced about threefold relative to pAKV6CAT in NIH 3T3 cells, and overexpression of ALF1 had no positive influence on CAT expression (Table 2).

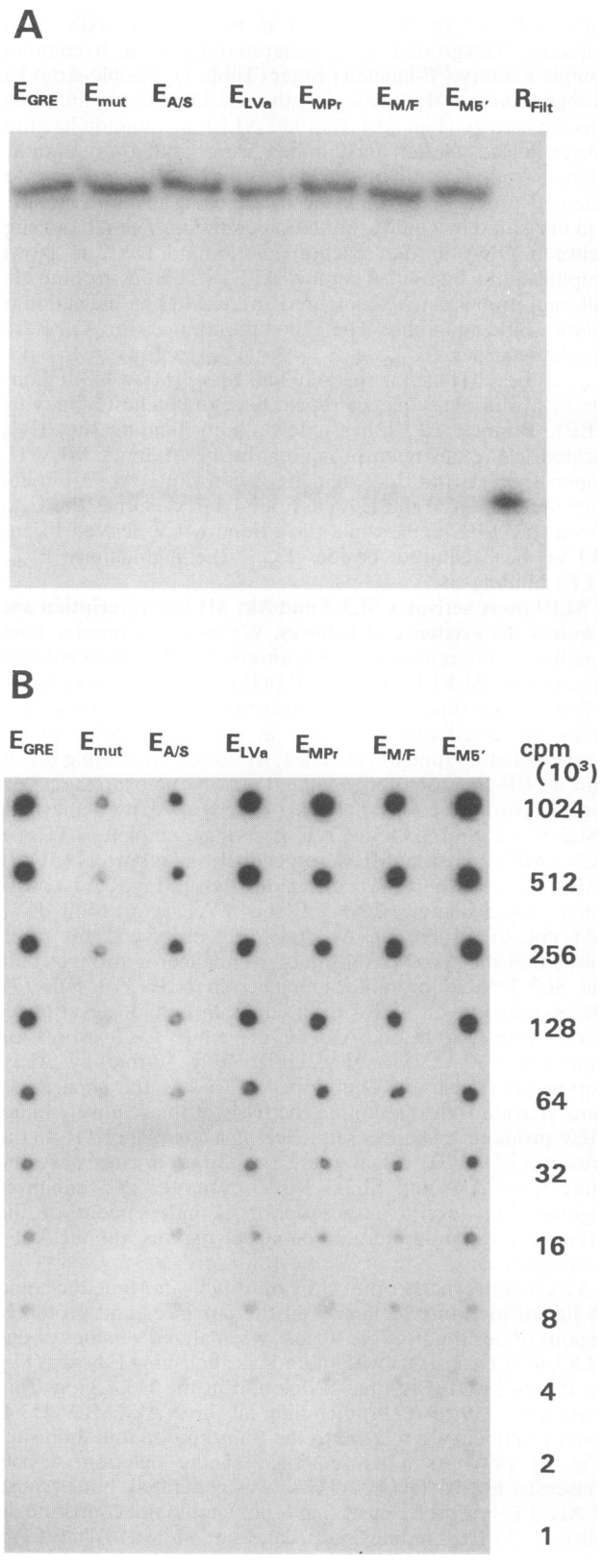

FIG. 4. ALF1 has variable affinity for murine type C retrovirus E boxes. (A) Denaturing PAGE of double-stranded ^{32}P-labeled oligonucleotides used in the nitrocellulose filter binding assay. R_{Filt} indicates the 5′-labeled primer used to generate the double-stranded E-box oligonucleotides. (B) *E. coli*-produced ALF1B was dotted on nitrocellulose filters and probed with the various E-box-containing ^{32}P-labeled oligonucleotides in concentrations ranging from 1×10^3 to $1{,}024 \times 10^3$ cpm. The type of E box included in each oligonucleotide probe is indicated at the top. (C) The amount of probe retained by ALF1 was determined by scintillation counting. Mean values for the amount of probe retained by ALF1 for each probe concentration were estimated from results of six experiments. Mean values have a standard deviation of less than 25%. The estimated mean values were plotted against the amount of probe in the reaction showing the relative affinity of ALF1 for each type of E-box probe.

Thus, *trans* activation of transcription by ALF1 requires a region in the Akv MLV U3 that includes the intact natural E-box sequences. Moreover, the experiment shows that plasmid sequences and the remaining segment of the Akv MLV LTR and LTR flanking sequences cannot replace the deleted Akv MLV U3 sequences in mediating *trans* activation by ALF1.

The deletion in pD19CAT removed an array of putative transcription factor binding sites beside the E boxes. For a more precise definition of sequence requirements for ALF1 *trans*-activator function, we introduced mutations by PCR into the two E_{GRE}s and the $E_{A/S}$ of pAKV6CAT, thereby generating pAKV(Eabc)CAT (Fig. 5). The introduced E-box mutations abolished the binding of ALF1 in vitro, as seen in EMSA and filter binding assays (Fig. 2A and B, lanes 4; Fig. 4). CAT expression from pAKV(Eabc)CAT in NIH 3T3 cells was similar to CAT expression from the parental pAKV6CAT (Table 2). Hence, in NIH 3T3 cells, the basal transcriptional activities of pAKV6CAT and pAKV(E-abc)CAT seem to be determined by transcription factors other than the class A bHLH E-box-binding factors such as ALF1. This is consistent with the lack of any detectable homodimeric ALF1-like DNA binding activity in NIH 3T3 cells (Fig. 3). Cotransfection with the ALF1 expression vector did not result in stronger CAT expression from pAKV(Eabc)CAT, whereas CAT expression

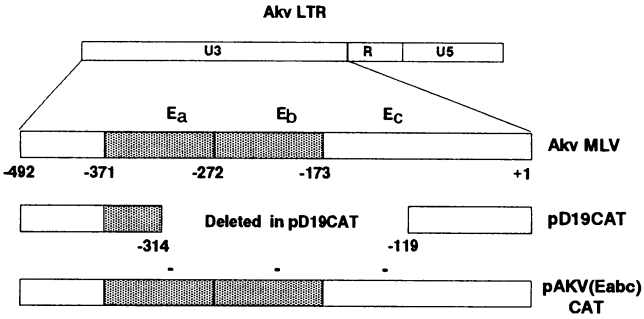

FIG. 5. Maps of the LTRs of Akv MLV, pD19CAT, and pAKV (Eabc)CAT. The sequences are indicated by boxes, with the repeated sequences in the U3 region shaded. The numbering of base pairs shown below the boxes is given relative to the cap site (+1). The positions of the various E-box sequences are given above the boxes. E_a and E_b are E_{GRE} sequences; E_c is an $E_{A/S}$ sequence (see Fig. 1); dashes indicate mutated E-box sequences. The pD19CAT construct (49) was derived from the Akv MLV U3 by introduction of a deletion extending from −119 to −314. The pAKV(Eabc)CAT construct was derived from the Akv MLV U3 by introduction of mutations in the three E-box sequences E_a, E_b, and E_c (see Materials and Methods).

from the parental pAKV6CAT was increased roughly threefold (Table 2). Consequently, the existence of intact E-box sequences is required for ALF1 to function as a *trans* activator of MLV transcription. In addition, minimal promoters including intact E boxes can be transcriptionally activated by overexpression of ALF1 or HEB (24) (data not shown).

ALF1 and the glucocorticoid receptor do not activate Akv MLV transcription synergistically. The Akv MLV transcriptional rate is activated by the steroid hormone dexamethasone in AKSL2 and L691 T cells as well as HeLa cells (8, 9, 17). Dexamethasone acts in a complex with the glucocorticoid receptor, which mediates transcriptional *trans* activation through binding to the GRE. We transfected NIH 3T3 cells with pAKV6CAT or pAKV(E-abc)CAT to examine whether the glucocorticoid receptor and ALF1 synergistically could *trans* activate transcription. Induction by dexamethasone increased CAT expression from pAKV6CAT to twice the level of CAT expression measured without dexamethasone induction (Table 3). CAT expression from pAKV6(Eabc)CAT was not induced by dexamethasone, showing that the mutations introduced in the E boxes abolished the GREs (Table 3). Mutation of the SL3-3 MLV E_{GRE} motifs also abolishes transcriptional activation by the glucocorticoid receptor (14). Transfection of the ALF1 expression vector followed by dexamethasone induction resulted in a 2.5-fold enhancement in CAT expression from pAKV6CAT and no enhancement from pAKV(Eabc)CAT (Table 3). As equivalent experiments without dexamethasone showed threefold enhancement in CAT expression from

TABLE 2. ALF1 *trans* activation requires E-box sequences

Construct	Mean CAT activity ± SD[a]	
	−ALF1	+ALF1
pAKV6CAT	100 ± 9	275 ± 41
pD19CAT	35 ± 4	30 ± 2
pAKV(Eabc)CAT	85 ± 13	80 ± 20
pSL3-3CAT	20 ± 5	210 ± 15

[a] Values represent a minimum of three transfection experiments and were normalized relative to the value for pAKV6CAT.

TABLE 3. Nonsynergistic function of ALF1 and glucocorticoid receptor in Akv MLV transcription

Plasmid	Mean CAT activity ± SD[a]			
	−Dex/ −ALF1	+1 μM Dex	+ALF1	+1 μM Dex/+ALF1
pAKV6CAT	100 ± 9	205 ± 51	275 ± 41	260 ± 21
pAKV(Eabc)CAT	85 ± 13	95 ± 14	80 ± 20	105 ± 17

[a] Values represent a minimum of three transfection experiments and were normalized relative to the value for pAKV6CAT. Dex, dexamethasone.

pAKV6CAT (Tables 2 and 3), ALF1 and the glucocorticoid receptor do not *trans* activate Akv MLV transcription in NIH 3T3 cells in an additive or synergistic manner. This finding is consistent with a noncompatible binding of the two transcription factors ALF1 and glucocorticoid receptor, which have overlapping binding sites in the U3 region of Akv MLV (9, 47).

ALF1 *trans* activates Moloney MLV transcription. Moloney MLV U3 has multiple E-box elements (Fig. 1). To monitor the function of ALF1 as a Moloney MLV transcriptional regulator, we carried out transfection analysis in NIH 3T3 fibroblasts, using several Moloney MLV *cat* gene reporter constructs provided by N. A. Speck (62). The construct pMoCAT carries Moloney MLV U3 sequences from the R region to just upstream of the repeats in U3 (62). Overexpression of ALF1 resulted in a fourfold increase in CAT expression from pMoCAT (Table 4). Therefore, ALF1 can *trans* activate transcription from the Moloney MLV U3 promoter-enhancer sequences.

To determine whether all of the Moloney MLV E boxes were involved in mediating the transcriptional response, we used a set of constructs generated by Speck et al. (62). Construct pMo(GREbc)CAT has mutations (GAACAGATG to TAAAAGATG) in the two promoter-proximal E_{GRE}s. The CAT expression directed by pMo(GREbc)CAT is fourfold lower than that measured for pMoCAT (62) (Table 4). Overexpression of ALF1 resulted in approximately a 15-fold enhancement of CAT expression, reaching the same level as from the ALF1-expressing parental pMoCAT construct (Table 4). The pMo(GREabc)CAT construct with mutations in all three E_{GRE}s has nearly a twofold-elevated CAT expression relative to pMoCAT. By ALF1 overexpression, pMo(GREabc)CAT reached a CAT expression level equivalent to that of the ALF1-expressing parental construct pMoCAT (Table 4).

The heavily deleted pMoPCAT construct has only the promoter-proximal Moloney MLV U3 sequences (from nucleotide −150) (62). The pMoPCAT construct mediates very low

TABLE 4. ALF1 mediates variable transcriptional responses

Group	Construct	Mean CAT activity ± SD[a]	
		−ALF1	+ALF1
A	pMoCAT	100 ± 7	425[b] ± 34
	pMo(GREbc)CAT	25 ± 6	350[b] ± 35
	pMo(GREabc)CAT	185 ± 54	490 ± 54
	pMoPCAT	2 ± 0.4	4[b] ± 1.2
B	pLFrCAT	100 ± 25	120 ± 35
	pLMoCAT	135 ± 11	605 ± 79
C	pFrLTRCAT	100 ± 14	90 ± 35
	pFrSSFVpCAT	75 ± 8	240 ± 60

[a] Values represent a minimum of three transfection experiments and were normalized relative to the value for pMoCAT in (group A), pLFrCAT (group B), and pFrLTRCAT (group C).

[b] An ALF1B expression vector was used.

basal CAT expression, and CAT expression was not dramatically increased by ALF1 overexpression (Table 4). Accordingly, pMoPCAT is not able to respond on ALF1 function as are the other Moloney MLV constructs. It should be noted that pMoPCAT has an E-box sequence, $E_{M/F}$, to which ALF1 may bind.

From the Moloney MLV experiments, we conclude that ALF1 can *trans* activate transcription from the Moloney MLV U3 region. As observed in the Akv and SL3-3 MLV experiments, Moloney MLV constructs are, with ALF1 overexpression, reaching a transcription level that is relatively independent of basal transcriptional activity. ALF1 can elevate transcription from Moloney MLV U3s with point mutations in the E_{GRE} subset of ALF1 binding sites. Thus, ALF1 may also *trans* activate transcription through the other E-box sites to which binding was observed, such as E_{LVa} and E_{MPr} (Fig. 4).

ALF1 *trans* activates Friend MLV transcription inefficiently but *trans* activates Friend SFFVp transcription. As the Friend MLV has only one putative ALF1 binding site in the U3 region, $E_{M/F}$, the capacity of ALF1 to *trans* activate transcription was examined. As reporter constructs, we used pLFrCAT, which has most of the Friend MLV U3 and R regions linked to the *cat* gene, or the exactly equivalent Moloney MLV construct pLMoCAT (constructs were provided by J. Lenz) (59). CAT expression levels from pLFrCAT and pLMoCAT were equivalent in NIH 3T3 cells (Table 4). Overexpression of ALF1 resulted in a 4.5-fold increase in CAT expression from pLMoCAT, while CAT expression from pLFrCAT was increased by a factor of 1.2 by ALF1 overexpression (Table 4). Thus, ALF1 cannot efficiently *trans* activate transcription on the background of Friend MLV U3 sequences.

We also examined whether the very low level of *trans* activation of Friend MLV by ALF1 could be observed on the background of a complete Friend MLV LTR as well. We used as the reporter construct pFrLTRCAT (provided by D. Kabat) (63), with a complete LTR surrounded by partial *env* and *gag-pol* sequences and with the *cat* gene linked to the *pol* sequences. CAT expression from pFrLTRCAT was 20% of the CAT expression from pLFrCAT (Table 4 and data not shown). Overexpression of ALF1 did not result in a significant increase in CAT expression from pFrLTRCAT (Table 4). We conclude that ALF1 cannot efficiently *trans* activate transcription on the background of Friend MLV sequences. Thus, ALF1 may not have a function in vivo as a transcription factor for Friend MLV.

The LTR of Friend SFFVp is thought to be derived from Friend MLV (69). The major difference between Friend MLV and Friend SFFVp LTRs is that the promoter proximal of the imperfect direct repeats in the Friend MLV U3 is deleted in the Friend SFFVp U3 (71). Interestingly, this deletion generates an E-box motif equivalent to the E_{GRE}. The existence of E_{GRE}s in various Friend SFFVs and Friend MCFVs seems to be general, suggesting a putative role in leukemogenesis (63, 71). To examine whether the existence of the additional E box in the Friend SFFVp U3 influences transcriptional *trans* activation by ALF1, we used the construct pFrSFFVpCAT (provided by D. Kabat) (63). This construct is related to pFrLTRCAT and includes equivalently a complete LTR surrounded by *env* and *gag-pol* sequences and the *cat* gene fused to *pol* sequences. In NIH 3T3 cells, the basal levels of CAT expression from pFrLTRCAT and pFrSFFVpCAT were shown to be equivalent (Table 4) (63). By ALF1 overexpression, the CAT expression from pFrSFFVpCAT was elevated threefold (Table 4). Thus, ALF1 has the capacity to function as an efficient transcriptional *trans* activator on the background of a complete Friend SFFVp LTR sequence.

DISCUSSION

Individual murine type C retroviruses possess a cell-type-restricted pattern of transcription. Cell-type-specific transcription may be explained by the presence of *cis* sequences located in the U3, which is the target for positively and negatively acting transcription factors. U3 sequences of murine type C retroviruses are highly related, but major divergences exist in the enhancer regions. The consensus E-box motif that overlaps the GREs is a relatively conserved sequence in enhancers of murine type C retroviruses. In this study, we show that ALF1 bHLH proteins can mediate transcriptional *trans* activation through complete LTR regions with an E-box requirement. ALF1 *trans* activates the murine type C retroviruses Akv, SL3-3, and Moloney MLVs as well as Friend SFFVp.

The U3 regions of murine type C retroviruses contain an array of E-box consensus sequences (18). ALF1 binds to these naturally occurring E boxes with different affinities (Fig. 4). ALF1 binds with relatively high affinity to the enhancer-located E_{GRE}s in Moloney, SL3-3, and Akv MLVs as well as Friend SFFVp and to E_{M5} in Moloney MLV. Intermediate binding affinity to E_{MPr} and E_{Lva} in the Moloney MLV and to $E_{M/F}$ in Friend SFFVp, Moloney MLV, and Friend MLV was observed. The weakest-affinity ALF1 binding site was $E_{A/S}$ in Akv and SL3-3 MLVs. The binding studies emphasize the importance of sequences flanking the E-box consensus core and show that ALF1 can distinguish between E boxes that are only slightly different in sequence (24). For example, E_{GRE}, $E_{A/S}$, E_{Mpr}, and $E_{M/F}$ boxes differ only in the four bases flanking the E-box core shown as N's in the sequence NNCAGATGNN. Bases further away from the E-box core do not seem to influence ALF1 DNA binding (data not shown). The preferences in ALF1 binding suggest that a putative function may be mediated mainly through binding to enhancer E boxes, as these sites seem to have the highest affinity. It should be noted that ALF1 in vivo may function as a heterodimer with other class A or class B bHLH proteins, which may modulate the E-box preferences (23, 24). Notably, a heterodimeric protein complex between HEB and the class B bHLH protein TAL1, which is proposed to be a critical factor in T-cell leukemogenesis, has a DNA sequence identical to that of E_{GRE} as a preferred binding site (23).

Transcriptional induction of Akv MLV by the hormone dexamethasone was prevented by the mutation damaging the E_{GRE} motifs, showing that the glucocorticoid receptor for mediating *trans* activation required the same DNA regions in the Akv MLV enhancer as does ALF1 (Table 3). This finding is in agreement with observations showing that a mutation abolishing the SL3-3 MLV E_{GRE} motifs also destroyed transcriptional induction by dexamethasone (14). Induction of the glucocorticoid receptor in conjunction with ALF1 overexpression resulted in no synergistic activity between these two transcription factors on the background of Akv MLV LTR sequences (Table 3). Thus, it is likely that the glucocorticoid receptor and ALF1 bind noncompatibly to the E_{GRE} motif.

In NIH 3T3 fibroblasts, the presence of E boxes in the Akv MLV U3 does not seem to affect the transcriptional rate (Table 2). The most likely explanation is that NIH 3T3 fibroblasts are devoid of functional class A bHLH protein activity including ALF1. This is consistent with the absence of detectable homodimeric ALF1 DNA binding activity (Fig. 3). Accordingly, in NIH 3T3 cells, the transcriptional activity of Akv MLV may be determined by another scenario of transcription factors rather than class A bHLH transcription factors such as ALF1. In NIH 3T3 cells, transcription from Akv MLV is fourfold higher than transcription from SL3-3 MLV. ALF1

overexpression resulted in roughly equivalent transcriptional activities of SL3-3 and Akv MLV. Hence, when ALF1 is overexpressed, this alone may control the level of transcription from promoter and enhancer regions with adequate E-box sequences.

In other studies, E-box motifs have been observed to be involved in transcriptional regulation of lymphoid cell-expressed genes such as the genes for immunoglobulin and the CD4 receptor (32, 57). Protein complexes with class A bHLH protein characteristics have been found to bind the E_{GRE}, μE2, κE2, IEB1, and CD4 enhancer E boxes in lymphoid cells (2, 14, 26, 44, 57). The latter protein complex was found to include HEB (57). The importance of murine type C retrovirus U3 E boxes in lymphoid cells is suggested by several studies. Corneliussen et al. (14) have found that nuclear protein complexes from lymphoid cells interact with the E_{GRE} and that mutation of the E_{GRE}s in a SL3-3 MLV U3 resulted in a half transcription rate in myeloma and T-cell lines. Speck et al. (61) have shown that mutations in the E_{GRE}s of Moloney MLV doubled the latency period of disease induction.

Friend MLV is transcriptionally weak in T cells, but the Friend MLV-related Moloney MLV is transcriptionally active in T cells. Opposite transcriptional preferences are observed in erythroid cells (59). The enhancer of Friend MLV includes most of the motifs determined to be important in Moloney MLV for generating T-cell transcriptional preference. These motifs are the LVb element with an overlapping Ets protein binding site and the enhancer core sequence (38, 61). Interestingly, Friend MLV has only one E-box motif, $E_{M/F}$, in the U3 region (30). The existence of a single E box in Friend MLV U3 relative to the eight E boxes in Moloney MLV U3 is striking considering the large sequence homology between the two U3 regions (18). This finding suggests that E-box-binding transcription factors may be involved in generating the transcription factor pattern on MLV U3 regions, which is involved in mediating the transcriptional differences between Moloney and Friend MLVs. In vivo, the high number of ALF1 binding sites in Moloney MLV U3 may ensure a high local concentration of ALF1 or other class A bHLH proteins at the promoter. Thereby, Moloney MLV should have a high level of accessibility to the transcriptional regulator function of ALF1 and other class A bHLH proteins. In NIH 3T3 cells, the transcriptional activities of Moloney and Friend MLV U3 regions are almost equivalent (Table 4). Our analyses demonstrate that ALF1 elevates transcription from the Moloney U3 region, while Friend MLV transcription is not dramatically affected by ALF1 either in constructs with the U3 region or in constructs with the complete LTR. In this way, Moloney MLV transcription seems to be more capable than Friend MLV transcription of responding positively to ALF1. This suggests that ALF1 may be involved in establishing differences in MLV transcriptional activity.

The U3 regions of leukemogenic Friend SFFVs and Friend MCFVs contain E_{GRE} motifs which do not exist in the parental Friend MLV (30, 71). In our analyses, we found that ALF1 could elevate transcription from an intact Friend SFFVp LTR, while no activation from the intact Friend MLV LTR was observed. Thus, ALF1 may in vivo function as a transcriptional activator for Friend SFFVp. We have no explanation of biological importance of this activation, as Friend SFFVp and Friend MLV seem to have the same pattern of cell type transcription (59, 63). Friend SFFVp in complex with its helper virus Friend MLV induces erythroleukemia with a much shorter latency period than Friend MLV alone. The major determinant for Friend SFFVp leukemogenic potential has been mapped to the protein-coding regions (36, 55). However, the existence of a minimum of one E_{GRE} in Friend SFFVs implies that the E box may have importance in the expression of viral genes in Friend SFFV leukemogenesis (71).

Synergistic functions between E-box-binding transcription factors and Ets transcription factors have been observed previously (53). Murine type C retroviruses may have adopted an equivalent transcriptional regulation. The enhancer of the immunoglobulin μ heavy-chain gene contains an Ets protein binding site, π (μA), next to the E_{GRE}-like E2 boxes, μE5 and μE2 (45, 53). The E12 bHLH transcription factor binds to the μE5 and μE2 boxes (53, 64). Analyses showed that E12 and Ets transcription factors each elevated transcription to a very limited level from a construct in which catenated μE2 and π sites directed transcription (53). However, when E12 and Ets were coexpressed in the same cell, they synergistically activated transcription (53). A protein with characteristics of an Ets family member has been found to bind to the LVb and LVc sites in the Moloney MLV U3 region (38). The LVb and LVc sites are located adjacent to the enhancer core sites in MLVs (38). A synergistic binding of Ets-1 and the core-binding factor as observed in the T-cell receptor β-gene enhancer may function for MLVs (73). Thus, in lymphoid cell types, ALF1 or other class A bHLH proteins, Ets proteins, and enhancer core-binding factors may cause synergistic transcriptional *trans* activation of MLVs.

By showing that the lymphotropic SL3-3 and Moloney MLVs are transcriptionally *trans* activated by the bHLH protein ALF1, while the erythrotropic Friend MLV is not efficiently *trans* activated, our studies may help to elucidate the complex nature of murine retrovirus transcriptional regulation and pathogenesis.

ACKNOWLEDGMENTS

We thank Claus Bus and Ole Jensen for excellent technical assistance, N. A. Speck for donation of various Moloney MLV CAT constructs, J. Lenz for donation of Friend and Moloney MLV CAT constructs, and D. Kabat for donation of Friend MLV and Friend SFFVp CAT constructs.

This project was supported by the Danish Biotechnology Program, the Danish Natural Science Research Council, and the Novo Foundation.

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